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Original article

Endogenous, regulatory cysteine sulfenylation of ERK kinases in response to proliferative signals

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ABSTRACT

ERK-dependent signaling is key to many pathways through which extracellular signals are transduced into cell-fate decisions. One conundrum is the way in which disparate signals induce specific responses through a common, ERK-dependent kinase cascade. While studies have revealed intricate ways of controlling ERK signaling through spatiotemporal localization and phosphorylation dynamics, additional modes of ERK regulation undoubtedly remain to be discovered. We hypothesized that fine-tuning of ERK signaling could occur by cysteine oxidation. We report that ERK is actively and directly oxidized by signal-generated H_2O_2 during proliferative signaling, and that ERK oxidation occurs downstream of a variety of receptor classes tested in four cell lines. Furthermore, within the tested cell lines and proliferative signals, we observed that both activation loop-phosphorylated and non-phosphorylated ERK undergo sulfenylation in cells and that dynamics of ERK sulfenylation is dependent on the cell growth conditions prior to stimulation. We also tested the effect of endogenous ERK oxidation on kinase activity and report that phosphotransfer reactions are reversibly inhibited by oxidation by as much as 80–90%, underscoring the importance of considering this additional modification when assessing ERK activation in response to extracellular signals.

1. Introduction

Reactive oxygen species (ROS), while previously considered detrimental byproducts of respiration [1], are now recognized as significant contributors to cellular signaling [2–4]. An important milestone in support of ROS as regulators of cell signaling was the discovery in the mid-1990s by Sundaresan et al. that H_2O_2 generation was required to elicit appropriate proliferative responses to Platelet-Derived Growth Factor (PDGF) in primary rat vascular smooth muscle cells [5]. Roles for H_2O_2 as a signaling mediator have now been demonstrated in many cell types and in vivo models [6–8], including our recent work establishing the important role of H_2O_2 in the proliferative responses to lysophosphatidic acid (LPA), a bioactive lipid synthesized by ovarian and prostate cancer cells and cell lines [9,10]. ROS have also been implicated in diverse signaling processes such as migration by endothelial cells [11], stem cell pluripotency and differentiation [12], and chondrocyte response to fibronectin fragments [13].

Much work has been done by us and others in the field of redox signaling to identify targets of signal-generated ROS [14–17]. Various ROS, reactive nitrogen species (RNS), and reactive sulfur species (RSS) react with cysteine residues of proteins, modifying the structure, dynamics, and/or function of those proteins. The oxidation of a cysteine residue is often reversible [15,18], which sets up the oxidation of a target cysteine on a protein to be a molecular switch. This switch-like property of cysteines has enabled them to serve specific functions as redox sensors on proteins [19–22]. For example, a well-established example of a binary cysteine switch is in the protein tyrosine phosphatase (PTP) family of enzymes [23]. In this case, the reactive, low pKa cysteine at the active site can become oxidized by ROS, inactivating the phosphatase, thereby favoring a sustained phosphorylation response to stimuli [3,4,24].

Identifying novel proteins whose cysteines are oxidized in response to extracellular signals has been historically difficult due to the generally labile nature of oxidized cysteine products. One class of cysteine probes developed by our research team is based on dimedone, which alkylates sulfenic acid (R-SOH) [15,25], the intermediate, two-electron

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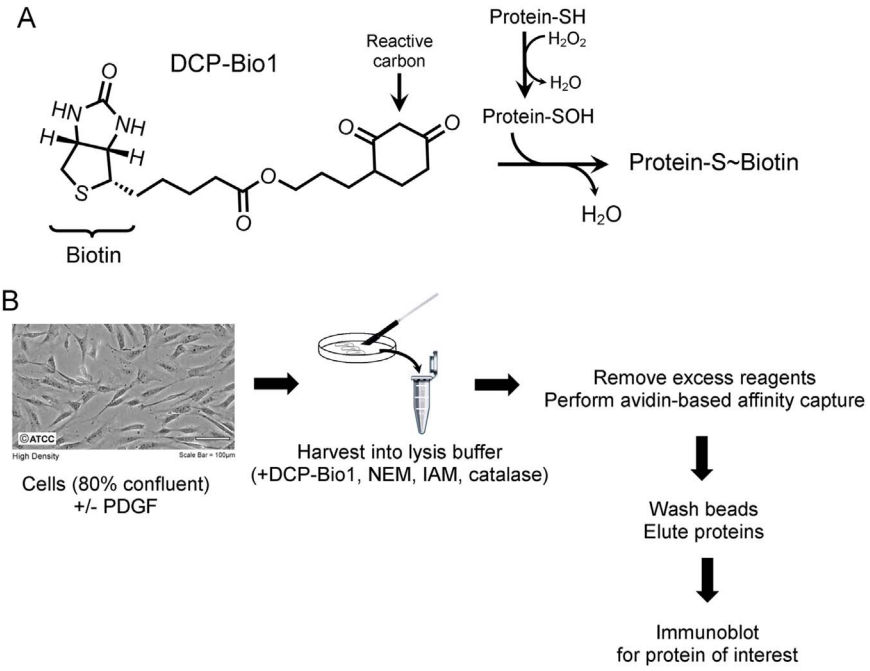

Fig. 1. Trapping of sulfenic acids within cellular proteins during lysis. A) Labeling of oxidized proteins with DCP-Bio1. The reactive carbon of DCP-Bio1 selectively reacts with sulfenic acids to form a covalent adduct, thereby biotinylating proteins containing sulfenic acids at the time of lysis. B) Overall experimental approach for labeling of proteins undergoing active oxidation to sulfenic acid. Cells are cultured to ~ 80% confluency, treated with an exogenous signal of interest such as platelet-derived growth factor (PDGF), and harvested in lysis buffer containing DCP-Bio1 to trap sulfenic acids, N-ethylmaleimide (NEM) and iodoacetamide (IAM) to alkylate free, reduced thiols (to minimize thiol oxidation during lysis), and catalase to remove any H_2O_2 formed during lysis. After incubation for 30 min on ice, lysates are clarified by centrifugation, excess DCP-Bio1 is removed with a molecular sieve (BioGel P6) spin column, and eluates are subjected to avidin-based affinity capture. Beads are then stringently washed to remove any unlabeled proteins carried over during capture. Captured proteins are eluted by incubation in 2% sodium dodecyl sulfate (SDS) at 100 °C for 10 min, followed by electrophoresis and immunoblotting for the protein of interest. To control for differences in capture efficiency and gel loading, a prebiotinylated bacterial protein, AhpC, is added to lysates at a ratio of 500 µg lysate to 0.5 µg AhpC before affinity capture. Photo courtesy of ATCC.

oxidized form of cysteine generated upon reaction with ROS molecules such as H_2O_2 (Fig. 1A). Using these dimedone-based probes to capture proteins undergoing active oxidation in cells responding to external signals can reveal the identity of the proteins oxidized in situ, and in favorable cases the specific cysteine residue undergoing oxidation [16,26].

One of the probes, DCP-Bio1 (Fig. 1A), contains the reactive core of dimedone linked to biotin, allowing for affinity capture of labeled cellular proteins undergoing active oxidation in cells (Fig. 1B) [27,28]. This probe has been used to identify multiple novel targets of oxidation, such as Akt2 [9,14,29], responding to extracellular signals [11,28,30,31]. Based on preliminary data from experiments suggesting that oxidation of mitogen-activated protein kinases ERK1 and ERK2 (designated ERK1/2) occurs during proliferation (unpublished) and migration [11], we undertook a project to further investigate the redox regulation of these proteins during cell signaling. ERK proteins are at the heart of a variety of signaling pathways involved in proliferation, differentiation, survival, migration [32,33], and at times even apoptosis [34,35]. This presents a conundrum of specificity – how do disparate signals induce specific responses while sharing the ERK signaling cascade? Evaluating ERK1/2 regulation is vital to understanding diseases from cancer to diabetes to neurological disorders. In fact, various components of the ERK1/2 signaling cascade (particularly Ras and Raf) are the most frequently found oncogenic mutations in human cancers, and an overall hyperactivation of the ERK1/2 pathway is reported in up to 90% of all human cancers [36].

Extensive research to date has revealed much about the ERK1/2 pathway and its regulation. Upon binding of an appropriate ligand to its receptor, a cascade of signaling protein activation occurs: the GTPase Ras activates the Raf kinases, which in turn activate the MAP kinase kinases MEK1 and MEK2 (referred to as MEK1/2). MEK1/2 proteins phosphorylate ERK1/2 at T183 and Y185 [32,37], hereafter designated as the TEY motif. This induces a conformational change that opens the active site of ERK1/2 [37,38] and enhances structural flexibility [39,40], allowing ERK1/2 to phosphorylate substrates. However, with over 300 known substrates in various subcellular compartments [36,41], multiple layers of regulation are involved, including temporal and spatial control of TEY phosphorylation and dephosphorylation [42,43]. While we know that dual phosphorylation is a prerequisite for ERK1/2 activation, it is not yet fully clear how cells orchestrate ERK1/2 localization, enable discrimination between substrates, or regulate the dephosphorylation of ERK1/2 by protein phosphatases [44].

Our knowledge of cysteine switches, including the tentative identification of ERK1/2 oxidation resulting from vascular endothelial growth factor (VEGF) signaling [11], led us to hypothesize that ERK1/2 cysteine oxidation could be an additional mode to control cell fates through ERK1/2 regulation. Human ERK1 and ERK2 contain 6 and 7 cysteines, respectively, half of which are solvent exposed and conserved in the MAPK family [37,45]. Analysis of the literature gives ample evidence that several of these cysteines could be redox sensitive. Under oxidative stress conditions, as mimicked by the bolus addition of H_2O_2 or nitric oxide donors, others have reported evidence for the modification of ERK1/2 cysteines [11,26,46–49]. ERK1/2 cysteine modification can also occur downstream of the physiological signals of vascular endothelial growth factor (VEGF) [11] and tumor necrosis factor α (TNF-α) [49], but it has not definitively been shown that ERK1/2 oxidation commonly occurs as a result of extracellular signals. Moreover, the linkage of signal-mediated oxidation events on ERK1/2 to their functional outputs has not been established. Here, we used the dimedone-based probe DCP-Bio1 in conjunction with immunoprecipitation and assays to test the redox sensitivity of recovered ERK1/2 activity, to assess function-altering ERK1/2 oxidation induced by extracellular stimuli. We report that ERK1/2 cysteine sulfenylation occurs in response to a variety of proliferative signals and that endogenous oxidation strongly modulates ERK1/2 kinase activity in vitro. To our knowledge, this is the first report to document the modification of ERK1/2 cysteines in response non-stress extracellular signals other than VEGF, and to relate the observed sulfenic acid formation to endogenous ERK1/2 kinase activity.

2. Materials and methods

2.1. Reagents and antibodies

Primary antibodies for Western blots to total ERK [recognizing both p44 (ERK1) and p42 (ERK2), RRID AB_390780], TEY-phosphorylated ERK1/2 (RRID: AB_331646), phospho-Elk1 (RRID: AB_2277933), along with anti-rabbit (RRID: AB_2099233) and anti-mouse (RRID: AB_330924) HRP-conjugated secondary antibodies, and Platelet Derived Growth Factor BB (abbreviated PDGF herein) were from Cell Signaling